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Abstract: Tumor cell-derived selectin ligands mediate contact to the endothelium, platelets, and leukocytes through binding to selectins that facilitates metastasis. Here, we describe the mechanism of how endogenous (non-tumor derived) selectin ligands contribute to metastasis using (1,3)fucosyltransferase 7 (Fuc-TVII(-/-))-deficient mice. Experimental metastasis of MC-38GFP and Lewis lung (3LL) carcinoma cells was attenuated in Fuc-TVII(-/-) mice, which express minimal amount of selectin ligands. We show that metastasis is dependent on selectin ligands carried on hematopoietic cells. P-selectin glycoprotein ligand-1 (PSGL-1) was identified as the major ligand facilitating monocyte accumulation at metastatic sites. Reduced recruitment of monocytes to metastasizing tumor cells in Fuc-TVII(-/-) mice correlated with attenuated metastasis. Adoptive transfer of Fuc-T7(+) monocytes rescued metastasis in Fuc-TVII(-/-) mice, indicating that selectin ligand-dependent recruitment of monocytes is required for cancer progression. Cytokine analysis in metastatic lungs revealed high expression of CCL2 in C57BL/6 mice that was significantly lower in Fuc-TVII(-/-) mice. The absence of monocyte recruitment in Fuc-TVII(-/-) mice correlated with increased apoptosis of tumor cells. Thus, the recruitment of monocytes to metastasizing tumor cells is facilitated by endogenous selectin ligands on monocytes that enable efficient tumor cell survival, extravasation, and metastasis.

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Metastatic growth progression caused by PSGL-1-mediated recruitment of monocytes to metastatic sites

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Abstract

Tumor cell-derived selectin ligands mediate contact to the endothelium, platelets, and leukocytes through binding to selectins that facilitates metastasis. Here we describe the mechanism how endogenous (non-tumor derived) selectin ligands contribute to metastasis using $\alpha(1,3)$ fucosyltransferase 7 (*Fuc-TVII*^{-/-}) deficient mice. Experimental metastasis of MC-38GFP and 3LL carcinoma cells was attenuated in *Fuc-TVII*^{-/-} mice which express minimal amount of selectin ligands. We show that metastasis is dependent on selectin ligands carried on hematopoietic cells. P-selectin glycoprotein ligand-1 (PSGL-1) was identified as the major ligand facilitating monocyte accumulation at metastatic sites. Reduced recruitment of monocytes to metastasizing tumor cells in *Fuc-TVII*^{-/-} mice correlated with attenuated metastasis. Adoptive transfer of Fuc-T7⁺ monocytes rescued metastasis in *Fuc-TVII*^{-/-} mice indicating that selectin ligand-dependent recruitment of monocytes is required for cancer progression. Cytokine analysis in metastatic lungs revealed high expression of CCL2 in C57BL/6 mice that was significantly lower in *Fuc-TVII*^{-/-} mice. The absence of monocyte recruitment in *Fuc-TVII*^{-/-} mice correlated with increased apoptosis of tumor cells. Thus, the recruitment of monocytes to metastasizing tumor cells is facilitated by endogenous selectin ligands on monocytes that enable efficient tumor cell survival, extravasation and metastasis.

Introduction

During hematogenous metastasis circulating tumor cells interact with blood constituents (platelets and leukocytes), which modulate their capacity to adhere to endothelium and to leave blood circulation. Malignant transformation is associated with alteration of cell surface glycosylation and presentation of altered glycan structures (1, 2). Furthermore, the enhanced expression of sialyl-Lewis^x (sLe^x) and/or sialyl-Lewis^a (sLe^a) is frequently associated with poor prognosis of carcinoma patients due to metastasis (3-5).

There is substantial evidence that selectins contribute to metastatic spread through mediation of tumor cell interaction within the circulation (reviewed in (1, 6, 7). Selectins are a family of cell adhesion molecules, expressed on activated endothelium (E- and P-selectin), leukocytes (L-selectin) and on activated platelets (P-selectin) which are involved in different physiological situations ranging from thrombosis, inflammation, hemostasis to cancer (1, 8). In the context of cancer progression, P-selectin facilitates platelet-tumor cell thrombi formation (9), L-selectin mediates leukocyte recruitment (10), and E- and P-selectin are required for adhesion of tumor cells to endothelium (11-13). The direct involvement of tumor cell-derived selectin ligands in metastasis has been shown both *in vitro* and *in vivo* (14-17). Enzymatic removal of selectin ligands, carcinoma mucins, resulted in reduced selectin ligand interactions and subsequently attenuation of metastasis (17). However, there is limited data on the involvement of endogenous (non-tumor derived) selectin ligands during cancer development and metastasis.

Selectins mediate the recruitment of leukocytes to inflammatory sites through binding to endogenous selectin ligands, expressed both on leukocytes, in high endothelial venules or on the activated endothelium (18). There are two major glycoproteins, P-

selectin-glycoprotein ligand-1; PSGL-1 and E-selectin ligand-1; ESL-1, which has been identified to mediate leukocyte rolling and adhesion during inflammation (19, 20). Selectin-mediated leukocyte trafficking is controlled prominently but not exclusively by $\alpha(1,3)$ fucosyltransferase Fuc-T7-dependent fucosylation, which is further complemented by $\alpha(1,3)$ fucosyltransferase Fuc-T4 activity (21, 22). In addition to homeostasis, leukocyte infiltration is also closely linked to cancer development. However, the mechanism of leukocyte recruitment is not unique for cancer progression and has many parallels with inflammation (8, 23). In particular, initiation of metastasis is dependent on the recruitment of leukocytes that is mediated by chemokines (24, 25). Tumor-derived CCL2 expression drives the recruitment of inflammatory monocytes that facilitates tumor cell extravasation and thereby metastasis (26-29). Yet, the mechanism of leukocyte adhesion and recruitment to metastatic tumors remains to be identified. Previously, we have shown that leukocyte recruitment to metastatic sites was L-selectin-dependent, indicating the active role of selectin in cancer progression (10). Furthermore, increased L-selectin ligand expression was detected in the vicinity of metastasizing tumor cells, but the identity of cells and the nature of the ligands remained unclear. The present study aims to identify selectin ligand-expressing cells and to elucidate their role in metastasis using Fucosyltransferase 7-deficient mice that are lacking the majority of selectin-ligands.

Material and Methods

Mice

Animals were maintained under specific pathogen-free conditions, and experiments were according to the guidelines of the Swiss Animal Protection Law, and approved by Veterinary Office of Kanton Zurich. C57BL/6 mice were purchased from the Jackson Laboratory. Fucosyltransferase 7-deficient mice (*Fuc-TVII*^{-/-}) and Fucosyltransferase 4 and 7 double deficient mice (*Fuc-TIV*^{-/-}/*Fuc-TVII*^{-/-}) in a C57BL/6 background were kindly provided by Dr. J.B. Lowe (University of Michigan, Ann Arbor, MI) and bred in house.

Cell culture

Mouse colon carcinoma cell line MC-38 stably expressing GFP (MC-38GFP) was grown in DMEM medium with 10% FCS (30). Lewis lung carcinoma cells (3LL) were grown in RPMI medium with 10% FCS (31). B16-BL6 melanoma cells were grown in DMEM medium with 10% FCS as described previously Hostettler (32).

Quantification of metastasis

Mice were intravenously (i.v.) injected with MC-38GFP cells (3×10^5) and euthanized after 28 days. Metastatic foci were counted and macroscopic pictures of lungs were documented. 3LL cells (1.5×10^5) were i.v. injected and tumor nodules were counted on day 14.

Histology and immunohistochemistry

Lungs fixed in 4% paraformaldehyde were embedded in paraffin blocks. Lungs sections (2 μ m) were stained with Hematoxylin/Eosin or various antibodies: Ki67 (Cl. SP6, NeoMarkers), anti-CD3 (Cl. SP7, NeoMarkers), anti-F4/80 (Serotec), anti-Ly6G (Becton

Dickinson (BD), anti-B220 (BD). For apoptosis assay anti-Caspase-3 (Cell Signaling) and anti-GFP (Fitzgerald Industries Int.) antibodies were used. Staining was performed on a NEXES immuno-histochemistry robot (Ventana instruments, Switzerland) using an IVIEW DAB Detection Kit (Ventana) or on a Bond MAX (Leica). Images were digitalized on Zeiss Mirax Midi Slide Scanner and analyzed with Mirax Viewer 1.12. software.

Bone marrow reconstitutions

C57BL/6 and *Fuc-TVII*^{-/-} mice were used for generation of bone marrow chimeras. Recipient animals were irradiated with 9 Gy in one dose and reconstituted by i.v. injection of 1×10^7 bone marrow cells (isolated from femur and tibia). Reconstitution efficiency was analyzed by the presence of selectin ligands in the peripheral blood leukocytes and the amount Ly6G⁺ cells in the blood 6-7 weeks after reconstitution.

Flow cytometry

Mice were perfused with PBS. Lungs were minced and digested with 2 mg/ml Collagenase D (Roche) and 2 mg/ml Collagenase A (Roche) for 1 h at 37°C. Cells were separated using 40 µm cell strainers. Red blood cells were lysed using PharmLyse (BD). Cells were incubated with anti-mouse CD16/32 mAb (BD) and stained with fluorophore-conjugated antibodies against: CD45, CD11b, F4/80, Ly6G, Ly6C, PSGL-1 (BD). Rabbit anti-ESL-1 serum VE12 (a generous gift from Dietmar Vestweber, Germany) was used together with donkey anti-rabbit-PE (eBiosciences). Anti-CD31-FITC antibody (Invitrogen) was used as an internal control for lung digestion. Blood samples were treated with PharmLyse (BD) and stained as described above. Selectin ligands were detected with mouse selectin-Fc chimeras (L-, P-, E-selectin; 10 µg/ml) that were pre-complexed with biotinylated goat-anti-human antibody (1:100; Sigma-Aldrich). Selectin binding was detected with Streptavidin-CyChrome (BD). Control samples were

incubated with selectin chimera in the presence of 10 mM EDTA. Data were acquired on a BD FACSCanto II flow cytometer (BD) and analyzed using Flow Jo software (Tree Star).

Selectin immune-precipitation and Western blot

Bone marrow was isolated from femurs and tibia of 6-8 weeks old C57BL/6 and *Fuc-TVII*^{-/-} mice. Cells were stained with CD11b, Ly6G and Ly6C antibodies (all BD) and sorted on a FACS Aria III (BD). The equal amount of CD11b⁺Ly6C^{hi} monocytes (15x10⁶ cells) and CD11b⁺Ly6G⁺ granulocytes (23x10⁶ cells) was lysed in a buffer containing 20 mM Tris pH 7.8, 150 mM NaCl, 1x Complete protease inhibitor cocktail (Roche) and 0.5 % Triton X-100. Mouse E-selectin-Fc chimera was pre-complexed with Protein A Sepharose 4 Fast Flow beads (GE Healthcare) for 1 h. Cell lysates were incubated with E-selectin/beads complex in the presence of 3 mM CaCl₂ overnight at +4 °C. Beads were washed three times with PBS and bound ligands were eluted in Laemmli loading buffer, boiled for 10 min at 95 °C and separated on 7.5 % SDS-PAGE gel. After a transfer the PVDF membrane was incubated with the rat anti-mouse PSGL-1 (BD) or rabbit anti-ESL-1 serum VE12 and developed with ECL method (GE Healthcare).

Analysis of leukocyte-tumor cell association

Frozen lung sections (8-10 μm) prepared from C57BL/6 and *Fuc-TVII*^{-/-} mice injected with MC-38GFP cells were stained with the following antibodies: CD11b (BD), Ly6G (BD), F4/80 (AbD Serotec). Goat anti-rat-Alexa568 Ab (Invitrogen) was used to visualize signals with a fluorescence microscope. Tumor cells were counted and the percentage of tumor cells associated with leukocytes was determined. Images were acquired with a SP2 confocal microscope (Leica) of a total 5 μm in a z-axis and analyzed with Imaris Software (Bitplane).

Real-time-PCR

Total RNA was isolated from PBS-perfused and flash frozen lungs or sorted cells (monocytes and granulocytes) from lungs using RNeasy Mini Kit (Qiagen). The quantity and quality of the RNA was determined using a Nanodrop 2000 (Thermo Scientific). Purified RNA was reversely transcribed into single stranded cDNA using Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. PCR was performed in CFX-96 thermocycler (Biorad) using a SYBR Green JumpStart Taq Ready Mix (Sigma-Aldrich) and primers specific for murine GAPDH, CCL2, CCL5, IL1 β , TNF α , TGF β . Primers (Microsynth; Switzerland) are listed in the supplementary Table S1. Expression levels of target genes were normalized to the housekeeping gene *GAPDH*. Relative changes in gene expression were calculated using the $2^{-\Delta\Delta C_t}$ method (33).

Isolation of monocytes

Bone marrow cell were harvested from 6-8 week old mice by flushing femur and tibia with PBS containing 2% FCS and 2.5 mM EDTA. Red blood cells were lysed using ammonium chloride solution. Bone marrow nucleated cells were pre-enriched using the MACS system. Briefly, cells were stained with biotinylated M-CSFR antibody (Cl. AFS98, Biolegend) and incubated with streptavidin-conjugated magnetic beads (Miltenyi Biotec). After MACS enrichment, cells were stained with Gr-1-PE mAb (Cl. RB6-8C5, eBioscience) and M-CSFR⁺ cells were stained with streptavidin-APC (eBioscience). Bone marrow monocytes were sorted as M-CSFR⁺Gr-1^{med} cells using a FACS Aria III cell sorter (BD).

Adoptive transfer

Fuc-TVII^{-/-} mice were intravenously injected with 2x10⁵ bone marrow monocytes isolated from C57BL/6 or *Fuc-TVII*^{-/-} mice, 6 h post-tumor cell injection (MC-38GFP or

3LL). Mice were euthanized after 28 days and metastasis was quantified as described above.

Statistical analysis

Statistical analysis was performed with the GraphPad Prism software (version 4.0). Data are presented as mean \pm SEM and were analyzed using the two-tailed Student's t-test.

Results

Endogenous selectin ligands facilitate metastasis

Fucosyltransferase-7 (Fuc-T7) is the major enzyme finalizing the synthesis of endogenous selectin ligands based on the core structure of sLe^x in mice (21, 22). To assess the role of selectin ligands during metastasis, we intravenously (i.v.) injected mouse colon carcinoma cells expressing GFP (MC-38GFP) in *Fuc-TVII*^{-/-} mice and metastasis were analyzed 28 days later. Significant reduction in the number of metastatic foci was observed in the lungs of *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice (Figure 1A-B). Immunohistological examination revealed no obvious differences in composition of Ki67⁺ (proliferating cells), F4/80⁺ (macrophages/monocytes) and Ly6G⁺ (neutrophils) cells within the tumors between *Fuc-TVII*^{-/-} and C57BL/6 lungs (Figure 1C). However, tumors in *Fuc-TVII*^{-/-} mice showed an enhanced infiltration of B220⁺ (B cells) and CD3⁺ T-cells. Of note, increased number of Ly6G⁺ cells was found in lung tissues of *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice. The significantly reduced number of metastatic nodules in *Fuc-TVII*^{-/-} mice indicated that the presence of endogenous (non-tumor-derived) selectin ligands is required for metastasis.

To test whether other tumor cells metastasize in endogenous selectin ligand-dependent manner, we injected Lewis lung carcinoma cells (3LL) and melanoma cells (B16-BL6) into *Fuc-TVII*^{-/-} mice. Similar to MC-38GFP cells, 3LL and B16-BL6 cells formed less metastatic nodules in *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice (Figure 1D-E, S1). There was no obvious difference in the composition of metastatic nodules as determined by immunohistochemistry (Figure S1C).

Selectin ligands on hematopoietic cells promote metastasis

To determine which selectin ligand-expressing cells promote metastasis, we generated chimeric mice. Reciprocal bone marrow reconstitutions (C57BL/6→*Fuc-TVII*^{-/-}; *Fuc-TVII*^{-/-}→C57BL/6) were performed and circulating myeloid cells (CD11b⁺ cells) were analyzed for the presence of selectin ligands 5 weeks later (Figure 2A-B and S2). We also confirmed increased levels of Ly6G⁺ cells in chimeric mice *Fuc-TVII*^{-/-}→C57BL/6 (Figure S2B-C) that was in line with the phenotype of *Fuc-TVII*^{-/-} mice (21). Chimeric mice were i.v. injected with MC-38GFP cells and the extent of metastasis was analyzed after 28 days (Figure 2C-D). *Fuc-TVII*^{-/-} mice expressing selectin ligands in the hematopoietic compartment (C57BL/6→*Fuc-TVII*^{-/-}) showed metastasis comparable to controls (C57BL/6→C57BL/6). On contrary, minimal metastasis was observed in chimeric mice expressing selectin ligands on the radio-resistant stromal compartment (*Fuc-TVII*^{-/-}→C57BL/6). These data demonstrate that selectin ligands on hematopoietic cells facilitate metastasis.

Reduced tumor cell survival in lungs of *Fuc-TVII* deficient mice

We next analyzed whether tumor cell seeding to the lungs is affected by endogenous selectin ligands. MC-38GFP-injected C57BL/6 and *Fuc-TVII*^{-/-} mice were terminated at

various time points and perfused lungs were evaluated for the presence of GFP⁺ tumor cells (Figure 3A-B). Similar amounts of MC-38GFP cells were observed at 30 min post-injection (p.i.) and their number decreased at 14 h and 24 h post-injection in both genotypes (Figure 3B). However a pronounced decrease of GFP⁺ cells was detected in lungs of *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice. Since the initial seeding/retention of tumor cells in the lungs was comparable and the difference in number of GFP⁺ tumor cells was detected first 14 h later, we tested the possibility that the endogenous selectin ligands might be required for tumor cell survival. We analyzed lungs of mice 24 h after tumor cell injection using Caspase 3 staining for detection of apoptotic cells, and GFP staining for living tumor cells (Figure 3C-D). We observed higher apoptosis of tumor cells in lungs of *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice, indicating that the absence of endogenous selectin ligands leads to a reduced tumor cell survival.

Endogenous selectin ligands facilitate monocyte recruitment and interactions with tumor cells

To test whether the observed enhanced tumor cell apoptosis in the absence of selectin ligands is due to the altered recruitment of leukocytes we analyzed lungs of C57BL/6 and *Fuc-TVII*^{-/-} mice injected with MC-38GFP cells for leukocyte infiltration at 14 h and 24 h p.i. (Figure 4 and S3A). Interestingly, significantly higher number of leukocytes (CD45⁺ cells) was detected in lungs of untreated *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice. The analysis of leukocyte subpopulations revealed a 5-fold higher presence of Ly6G⁺ cells (neutrophils/granulocytes) in lungs of naïve *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice that was also confirmed by immunohistochemical analysis (Figure S3B). Tumor cell injection increased CD45⁺ cell infiltration in both genotypes. While there was no change in number of Ly6G⁺ cells in C57BL/6 mice, further increase in *Fuc-TVII*^{-/-} mice

24 h p.i. was detected. The higher numbers of Ly6G⁺ cells observed in lungs of *Fuc-TVII*^{-/-} mice seemed to be independent of selectin ligand expression. On contrary, a 3-fold increase in inflammatory monocytes (Ly6C^{hi} cells) was detected in lungs of C57BL/6 mice 14 h p.i. (Figure 4). The numbers of Ly6C^{hi} cells in *Fuc-TVII*^{-/-} mice remained on the same albeit higher level regardless of tumor cell injection. Accordingly, we observed significantly higher numbers of F4/80⁺ (macrophages) cells in C57BL/6 mice. These data showed that tumor cell-induced infiltration of Ly6C^{hi} cells to the lungs is dependent on endogenous selectin ligands.

To assess whether overall changes in leukocyte infiltration to the lungs alter tumor cell–myeloid cell interactions during tissue colonization, we analyzed lung sections from MC-38GFP injected mice using immunohistochemistry. While the majority of tumor cells were associated with CD11b⁺ cells in C57BL/6 mice at 14 h p.i. (Figure 4E), there was minimal contact detected in *Fuc-TVII*^{-/-} mice. In addition, about 50% of tumor cells were associated with F4/80⁺ cells in C57BL/6 mice and the number increased at 24 h p.i. (Figure 4F). On contrary, tumor cells were minimally associated with F4/80⁺ cells in *Fuc-TVII*^{-/-} mice at any time point. Of note, despite the generally higher residency of Ly6G⁺ cells in the lungs of *Fuc-TVII*^{-/-} mice an increase in tumor cell association was observed only at 24 h p.i. (Figure 4F). The reduced recruitment of leukocytes to metastasizing tumor cells could be a result of missing local expression of vascular selectins. Therefore, we tested the expression of P- and E-selectin in the tumor microenvironment of *Fuc-TVII*^{-/-} mice at 6 h p.i. (Figure S3C). We observed comparable expression of both P- and E-selectin in the vicinity of tumor cells in the lungs of both mouse genotypes. Thus, the presence of endogenous selectin ligands on monocytes is required for the efficient recruitment of F4/80⁺ cells to tumor cells during metastatic initiation in the lungs.

PSGL-1 is the prominent selectin ligand on Ly6C^{hi} monocytes

We next analyzed the presence of known selectin ligands P-selectin glycoprotein ligand-1 (PSGL-1) and E-selectin ligand-1 (ESL-1) on granulocytes and monocytes from C57BL/6; *Fuc-TVII*^{-/-} and *Fuc-TIV*^{-/-}*Fuc-TVII*^{-/-} mice and the binding of selectins. Flow cytometry analysis revealed that both cell population (monocytes and granulocytes) express PSGL-1 and ESL-1 at similar levels, irrespective of mouse genotype (Figure 5A). A pronounced reduction of E- and P-selectin binding to *Fuc-TVII*^{-/-} monocytes and granulocytes was observed (Figure 5A and S4). Interestingly, the minimal presence of E-selectin ligands in *Fuc-TVII*^{-/-} monocytes (6%) and granulocytes (2%) was reduced to 0.7% and 0.6% in respective cell populations in *Fuc-TIV*^{-/-}*Fuc-TVII*^{-/-} cells. This finding indicated the presence of ESL-1. To determine which of the selectin ligands is affected by the absence of Fuc-T7 enzyme, we immune-precipitated selectin ligands using E-selectin chimera from monocytes and granulocytes either of C57BL/6 or *Fuc-TVII*^{-/-} mice. We observed a dominant PSGL-1 precipitation in C57BL/6 monocytes and granulocytes that were completely absent in respective cell populations isolated from *Fuc-TVII*^{-/-} mice (Figure 5B). However, ESL-1 has been detected both in monocytes and granulocytes albeit at significantly lower levels in *Fuc-TVII*^{-/-} cells compared to C57BL/6. Taken together these data strongly indicate that Fuc-T7-dependent fucosylation of PSGL-1 is responsible for the efficient monocyte recruitment to metastatic sites.

Monocytes carrying selectin ligands rescue metastasis in *Fuc-TVII*^{-/-} mice

Next we tested whether a single injection of monocytes carrying selectin ligands affect metastasis. *Fuc-TVII*^{-/-} mice were administered MC-38GFP cells that were followed by single injection of purified monocytes isolated from C57BL/6 or *Fuc-TVII*^{-/-} mice 6 h

later. Adoptive transfer of C57BL/6 monocytes restored metastasis in *Fuc-TVII*^{-/-} mice to levels comparable to C57BL/6 mice (Figure 6A-B). Similarly, adoptive transfer of monocytes rescued 3LL metastasis in *Fuc-TVII*^{-/-} mice (Figure S5). No effect on metastasis was observed after intravenous injection of selectin ligand-deficient *Fuc-TVII*^{-/-} monocytes. These data show that endogenous selectin ligands on monocytes are essential for their recruitment to the lungs and therefore sufficient to promote metastasis.

Inflammatory monocytes support tumor cell survival, growth and metastasis through secretion of cytokines and growth factors (23). To test whether reduced monocyte recruitment in *Fuc-TVII*^{-/-} mice alters the microenvironment of metastasizing tumor, we examined cytokines in the lungs of MC-38GFP-injected mice after 24 h (Figure 6C and S6). No significant changes in expression levels of IL1 β , TNF- α and CCL5 were observed in both genotypes (C57BL/6 and *Fuc-TVII*^{-/-} mice). The analysis of neutrophil-specific chemokines, CXCL1, CXCL2 and CXCL5, showed minimal changes in mice of both genotypes (data not shown) indicating that the general increase in lung neutrophils observed in *Fuc-TVII*^{-/-} mice is based on a different mechanism. Interestingly, the expression of CCL2 was 6-fold higher in C57BL/6 mice 14 h p.i. that remained 2-fold above the basal levels also at 24 h p.i (Figure 6C). In comparison, CCL2 expression levels were always reduced in *Fuc-TVII*^{-/-} mice albeit some induction after tumor cell injection has been detected. Of note, TGF β expression was slightly decreased in lungs of C57BL/6 mice injected with tumor cells 24 h p.i. compared to naïve lungs (Figure S6).

It was previously shown that CCL2 promotes tumor cell extravasation through the recruitment of monocytic cells (27, 28), and mediate a direct endothelial activation resulting in increased vascular permeability (28). Since we observed reduced CCL2 expression levels in lungs of *Fuc-TVII*^{-/-} mice we tested the lungs vascular permeability

upon tumor cell injection. A comparable vascular leakiness was detected in C57BL/6 and *Fuc-TVII*^{-/-} mice (Figure S7). This observation is in line with previous data showing that tumor cell-derived CCL2 is mainly responsible for induction of vascular permeability (28). Thus, lower levels of CCL2 in tumor cell-injected *Fuc-TVII*^{-/-} mice is likely the consequence of reduced monocyte recruitment to the lungs. To test this hypothesis we isolated monocytes and granulocytes from lungs of tumor cell-injected mice and analyzed CCL2 expression. Monocytes (CD11b⁺Ly6C^{hi}) isolated from lungs of *Fuc-TVII*^{-/-} and C56BL/6 mice had comparable levels of CCL2 expression that was about 4-fold higher than in circulating monocytes (Figure 6D). This finding indicated that monocytes recruited to the lungs express higher levels of CCL2. Of note, we detected minimal expression of CCL2 in granulocytes (CD11b⁺Ly6G^{hi}Ly6C^{medium}) from both genotypes indicating that neutrophils do not significantly contribute to CCL2 presence in the lungs. This data provided evidence that the absence of selectin ligands reduced the ability of monocytes to migrate to the metastatic lungs and resulted in attenuation of metastasis due to inefficient tumor cell extravasation

Discussion

Inflammatory leukocytes at primary tumor and metastatic sites contribute to formation of the pro-tumorigenic microenvironment through production of cytokines; growth factors and matrix-degrading enzymes (23, 34). Increased recruitment of myeloid cells and inflammatory monocytes is associated with enhanced tumor cell extravasation and malignant outgrowth of breast and colon cancers (26, 28, 35). In addition, leukocyte recruitment is dependent on the capacity of leukocytes to adhere to activated endothelium through L-selectin (10). Here we provide evidence that endogenous

selectin ligands on monocytes are required for their specific recruitment to metastatic sites. Furthermore, PSGL-1 was identified as the major selectin ligand responsible for the selectin-mediated recruitment of monocytes to metastatic sites. This is in agreement with previous published results that Fuc-T7 is the major fucosyltransferase responsible for the synthesis of selectin ligands on leukocytes as determined by selectin chimera binding to intact cells (21, 22). In addition, Fuc-T7 has been identified as the main enzyme modifying PSGL-1 while its absence had little effect on ESL-1 recognition by E-selectin (36). We also showed that E-selectin immune-precipitated ESL-1 both from C57BL/6 and *Fuc-TVII*^{-/-} cells, albeit at minimal levels. These findings corresponded well with the low detection of E-selectin ligands on cells from *Fuc-TVII*^{-/-} mice using flow cytometry. However, neutrophils from PSGL-1-deficient mice showed significant binding of E-selectin using flow cytometry (37). These observations are supported by a recent study where an additional knock-down of ESL-1 in PSGL-1 deficient neutrophils further reduces E-selectin binding (19). We cannot completely explain the discrepancy in E-selectin ligand detection on neutrophils that would correspond to ESL-1 ligands. However, it is possible that the complete absence of a major selectin ligand carrier, PSGL-1, induces altered presentation of selectin ligands on cell surfaces which may reflect enhanced E-selectin binding, thus ESL-1 detection. Accordingly, the absence of Fuc-T7 enzyme leads to significant reduction of overall fucosylation and thus reduced presence of selectin ligands as described in this study which is in agreement with previous studies (21, 22). It is accepted that only the presentation of selectin ligands on cell surface makes them “the real” ligands that are biologically relevant in physiological processes (38). Nevertheless, we provided evidence that Fuc-T7 enzyme activity is critical for the formation of selectin ligands on PSGL-1 that mediates recruitment of inflammatory monocytes (Ly6C^{hi}) to early metastatic sites. This finding is in agreement

with a recent identification of PSGL-1 ligands as mediators of monocyte recruitment to atherosclerotic lesions (39). Thus, similarly to inflammation, leukocyte recruitment to metastatic microenvironment is also determined by selectin ligands on leukocytes (40). Whether leukocytes are recruited through vascular selectins, P- and/or E-selectin, or through binding to L-selectin on already adherent leukocytes remain to be determined. An accelerated growth of primary tumors in selectin-deficient mice has been previously reported (41). Similarly, we observed an accelerated tumor growth of subcutaneously injected MC-38GFP cells in *Fuc-TVII*^{-/-} mice compared to C57BL/6 mice (Figure S8). This finding indicates that endogenous selectin ligands restrict primary tumor growth but potentiate metastatic outgrowth at metastatic sites. Therefore, selectin-mediated interactions have spatially and temporally-defined diverse functions during tumorigenesis. Interestingly, the accelerated tumor growth in E-, P- and L-selectin-triple deficient mice was dependent on bone marrow-derived cells (41). While we confirmed that selectin-dependent recruitment of monocytes facilitates metastasis, the identity and function of leukocytes recruited to primary subcutaneous tumors remains to be identified.

Neutrophils during cancer progression exert dual activities either promoting tumor cell growth or contributing to elimination of tumor cells depending on the cellular context and the polarization status of the cells (42, 43). Neutrophil-melanoma cell interactions promoted tumor cell retention within the lungs (42). In mesothelioma tumors neutrophils contributed to cancer progression, while the blockade of TGF- β resulted in recruitment of cytotoxic neutrophils leading to elimination of tumor cells (43). The observed higher numbers of lung-residing neutrophils and also their association with tumor cells in *Fuc-TVII*^{-/-} mice raised also the question whether neutrophils affect metastasis. Depletion of Ly6G cells resulted in a partial rescue of metastasis indicating

that neutrophils also contributed to elimination of tumor cells in *Fuc-TVII*^{-/-} mice (Figure S9). This finding was further corroborated by detection of apoptotic tumor cells in lungs of *Fuc-TVII*^{-/-} mice that was increased compared to C57BL/6 mice. Apparently, neutrophil recruitment to the lungs was not dependent on selectin ligands, which is in agreement with previous reports that neutrophils are able to infiltrate some organs including lungs in a selectin-selectin ligand independent mechanism (44).

Several studies reported that reduced recruitment of monocytes to tumor cells resulted in increased neutrophil association (28, 45). Monocyte recruitment to metastasizing tumor cells is primarily CCL2-dependent (28, 35, 45) and is normally associated with reduced infiltration of neutrophils (45). This plasticity in leukocyte recruitment corresponds with our findings where reduced monocyte recruitment was accompanied with lower CCL2 levels and resulted in enhanced neutrophil recruitment in *Fuc-TVII*^{-/-} mice. Nevertheless, the adoptive transfer of selectin-ligand-positive monocytes rescued metastasis that underlines the active role of monocytes during tumor cell extravasation (27, 28).

Elevated levels of CCL2 at metastatic sites correlated with enhanced metastasis of breast, colon, prostate and lung cancer cells in different mouse models (27-29). Here we provided evidence that inflammatory monocytes express significant levels of CCL2 that possibly contribute to higher CCL2 levels in lungs during metastasis.

In conclusion, the rescue of metastasis upon adoptive transfer of inflammatory monocytes confirmed the essential role of selectin-mediated interactions during metastatic initiation. This finding provided evidence that apart from well-known involvement of tumor cell-derived selectin ligands, the presence of endogenous selectin ligands, particularly PSGL-1 on leukocytes, are required for cancer progression.

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Figure Legends

Figure 1. Endogenous selectin ligands facilitate metastasis. **A)** Representative pictures of metastatic lungs from C57BL/6 (n=12) and *Fuc-TVII*^{-/-} (n=7) mice 28 days p.i. with MC-38GFP cells. **B)** Quantification of metastatic foci 28 days p.i. **C)** Histological analysis of lungs from C57BL/6 and *Fuc-TVII*^{-/-} mice 28 days p.i. with MC-38GFP cells. Scale bars are indicated. H&E: Hematoxylin & Eosin, Ki67: proliferation marker, F4/80: monocytes and macrophages, Ly6G: neutrophils, B220: B-cells, CD3: T cells. The insert in Ly6G⁺ cells shows magnification at the tissue-tumor interface at higher magnification. Scale bar = 100 μ m. **D)** Macroscopy of lungs from C57BL/6 and *Fuc-TVII*^{-/-} mice 14 days p.i. with Lewis lung carcinoma (3LL) cells. **E)** Quantification of tumor nodules (n = 4-5). ***, p < 0.001.

Figure 2. Selectin ligands on hematopoietic cells are required for metastasis. **A)** Quantification of selectin ligands on monocytes (Ly6C^{hi} cells) from peripheral blood of C57BL/6, *Fuc-TVII*^{-/-} and chimeric mice: C57BL/6 \rightarrow *Fuc-TVII*^{-/-} or *Fuc-TVII*^{-/-} \rightarrow C57BL/6 using L-selectin (n = 4). **B)** Representative histograms of E-, P- and L-selectin ligand expression on monocytes from the blood of control and chimeric mice. **C)** Macroscopy of lungs and **D)** quantification of metastatic foci in lungs of mice 28 days p.i. with MC-38GFP cells, respectively (n = 5/6). **, p < 0.01; ***, p < 0.001.

Figure 3. The absence of endogenous selectin ligands reduces tumor cell survival due to enhanced apoptosis. **A)** Gating strategy for the quantification of GFP-expressing tumor cells in lung homogenates of C57BL/6 and *Fuc-TVII*^{-/-} mice at various time-points. CD31⁺ endothelial cells (ECs) were used as an internal reference. **B)** Flow cytometry analysis of MC-38GFP cells in lung homogenates at different time points after injection.

The number of tumor cells is normalized to 20'000 ECs. **C)** Caspase 3 staining of lung sections from C57BL/6 and *Fuc-TVII*^{-/-} mice 24 h after MC-38GFP injection (n=3). Representative images of Caspase 3⁺ tumor cells in *Fuc-TVII*^{-/-} mice and Caspase 3⁺ tumor cells in C57BL/6 lungs. **D)** Analysis of GFP⁺ tumor cells in lungs of mice used for Caspase 3 staining. *, p < 0.05; ***, p < 0.001.

Figure 4. Fuc-TVII deficiency alters recruitment of leukocytes to the lungs. A-D)

Flow cytometry analysis of leukocytes from lung homogenates of untreated mice (C57BL/6, *Fuc-TVII*^{-/-}) and mice 14 h and 24 h p.i. with MC-38GFP cells (n = 4-6). **A)** The number of leukocytes (CD45⁺ cells) was normalized to 1'000 endothelial cells (ECs). Ly6C⁺ (**B**), Ly6G⁺ (**C**) and F4/80⁺ (**D**) cells are presented as percentage of CD45⁺ cells. **E-F)** Quantification of myeloid cell-tumor cell interactions in lungs of C57BL/6 and *Fuc-TVII*^{-/-} mice at 14 h and 24 h p.i. with MC-38GFP cells by confocal microscopy (n = 3). **G)** Representative confocal microscopy images of the contact of tumor cells (green) with F4/80⁺ or Ly6G⁺ cells (both in red) in C57BL/6 and *Fuc-TVII*^{-/-} lungs at 24 h p.i. Nuclei (blue) are stained with DAPI. Scale bar: 10 μm. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

Figure 5. PSGL-1 on monocytes is the prominent ligand for selectin-mediated recruitment of Ly6C^{hi} monocytes. A)

Gating strategy and the analysis of PSGL-1 and ESL-1 expression in the peripheral blood cells. Ly6C^{hi} monocytes and Ly6C⁺ granulocytes derived from C56BL/6, *Fuc-TVII*^{-/-} and *Fuc-TIV*^{-/-}/*Fuc-TVII*^{-/-} double deficient mice were stained with E-selectin. **B)** Immune-precipitation of E-selectin ligands on Ly6C^{hi} Monocytes and Ly6G⁺ granulocytes. E-selectin-bound proteins were separated on SDS-PAGE gel and blotted with either anti-PSGL-1 or ESL-1 antibodies. Splenocytes were used as a positive control.

Figure 6. Selectin ligand-expressing monocytes rescue metastasis in *Fuc-TVII*^{-/-} mice. Adoptive transfer of monocytes (monos) isolated from bone marrow of C57BL/6 and *Fuc-TVII*^{-/-} mice that were intravenously injected into *Fuc-TVII*^{-/-} mice 6 h p.i. of MC-38GFP cells. **A)** Macroscopy of lungs from *Fuc-TVII*^{-/-} mice that were injected with C57BL/6 or *Fuc-TVII*^{-/-} monocytes 28 days p.i. **B)** Quantification of tumor nodules in lungs at day 28 (n = 7-10). **C-D)** CCL2 expression in the whole lungs (**C**) and monocytes (Ly6C^{high}) isolated from lungs (**D**) of mice i.v. injected with MC-38GFP cells after 14 h and 24 h, respectively. Data represents two independent experiments. Expression levels were analyzed by real time-PCR and normalized to GAPDH. Purified blood monocytes (Ly6C^{high}) from untreated mice were used as a control. Data are presented as 2^{-ΔΔCt}. *, p < 0.05; ***, p < 0.001.